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PRINCIPAL INVESTIGATOR: Nandini Ghosh-Choudbury, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Health Science
Center at San Antonio
San Antonio, Texas 78284

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Nandini Ghosh-Choudbury, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas Health Science Center at San Antonio
San Antonio, Texas 78284

E-MAIL:
choudhury@uthscsa.edu

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13. ABSTRACT (Maximum 200 Words)
Bone morphogenetic protein-2 (BMP-2), a potential growth factor for osteogenesis, inhibited estrogen-responsive growth of MCF-7 breast cancer cells. The mechanism involved inhibiton of estrogen-induced mitogen activated protein kinase (MAPK) activity by BMP-2. BMP-2 also inhibited cell cycle progression in MCF-7 cells by increasing the level of a cyclin dependent kinase (CDK) inhibitor (CKI), namely, p21. This in turn resulted in inhibition of CDK activities which inhibited phosphorylation of retinobastoma protein (pRb). pRb phosphorylation is tightly linked with its activity in cell cycle progression. Thus by inhibiting pRb phosphorylation BMP-2 arrested the cell cycle at the G1 phase and thus inhibited uncontrolled proliferation of these cells. In order to extend this finding in a clinical background we are now establising animal models of human breast cancer cell growth in which we are going to express BMP-2 under a tetracyclin regulatable promoter in the breast cancer cells. We have generated cell lines using these expression plasmids and have started to characterize them. We will also analyze breast cancer tissue samples for the expression of BMP-2 and its receptors. Once the analysis is complete, we will categorize the cancer samples in terms of their growth and metastasis property. This will help us to find any correlation of expression of BMP-2 and its receptors with growth and metastatic behavior of breast cancer tissue samples. Role of BMP-2 in growth and metastatic property of breast cancer cells have not been studied before and our preliminary data suggest that this study can prove to be clinically important in future.

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FOREWORD

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Nandini Ghosh Choudhury, 6/13/00

PI - Signature   Date

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Table of Contents

INTRODUCTION:

The purpose of this research is to understand the mechanism of growth inhibition of breast cancer cells in response to bone morphogenetic protein-2 (BMP-2). Breast tumors grow very aggressively in the primary site (breast) as well as the secondary sites (lung, bone etc.). Estrogen is a potent mitogen for a number of breast tumors and thus estrogen promotes the tumor to grow even more rapidly. We have studied the growth of breast cancer cells *in vitro*, under various growth conditions. Our main purpose was to identify factors that would slow down the growth of these cells. BMP-2, a member of TGF β related growth factor superfamily, was found to inhibit growth of both estrogen responsive and non-responsive breast cancer cell growth. We have studied the underlying mechanism of this inhibition in cells from both of these categories and had found that the basic mechanism is same in both the cell lines. This finding makes BMP-2 a more clinically important candidate for controlling breast cancer cell growth. We are currently trying to reproduce our in vitro findings in animals.

BODY:

Task 1. **To correlate the levels of BMP-2 and BMP-2 receptor expression in breast cancer cell lines and tissue samples with their bone metastasis status. (months 1-12)**

- **Perform Northern as well as the RT-PCR analysis of BMP-2 and BMPR expression in different ER negative and ER positive cells and in different breast tumor tissue samples (months 1-6).**
- **Analyze the expression level of BMP-2 and BMPR different ER negative and ER positive cells and in different breast tumor tissue samples (months 6-7).**

Towards this goal I have isolated RNA from different breast cancer cell lines and performed RTPCR analysis using primers specific for BMP-2, BMP receptor IA and IB. Fig. 1 shows the results from these experiments. The cell lines marked FRC and FMC are osteoblast cells, used as a positive control for BMP-2, BMP2RIA and IB. I found that though the breast cancer cell lines express BMP-2 in detectable quantities, the expression of receptors were not abundant.

Currently, I am trying to confirm these findings using Northern analysis. Following the completion of analysis I will categorize these cell lines with respect to their estrogen receptor status and the expression of BMP-2 and its receptors by these cells.

I am in the process of getting tissue samples from the breast cancer tissue bank. Once I get the samples I will start making RNA from them and analyze for expression of BMP-2 and its receptors by Northern and RTPCR analysis.

- **Correlate the metastatic potential of the breast cancer cells using the animal model described above** *by in vivo* **study (months 6-12).**

- **Correlate metastatic property of the tissue samples with the expression levels of BMP-2 and BMPR in these samples (months 7-8).**
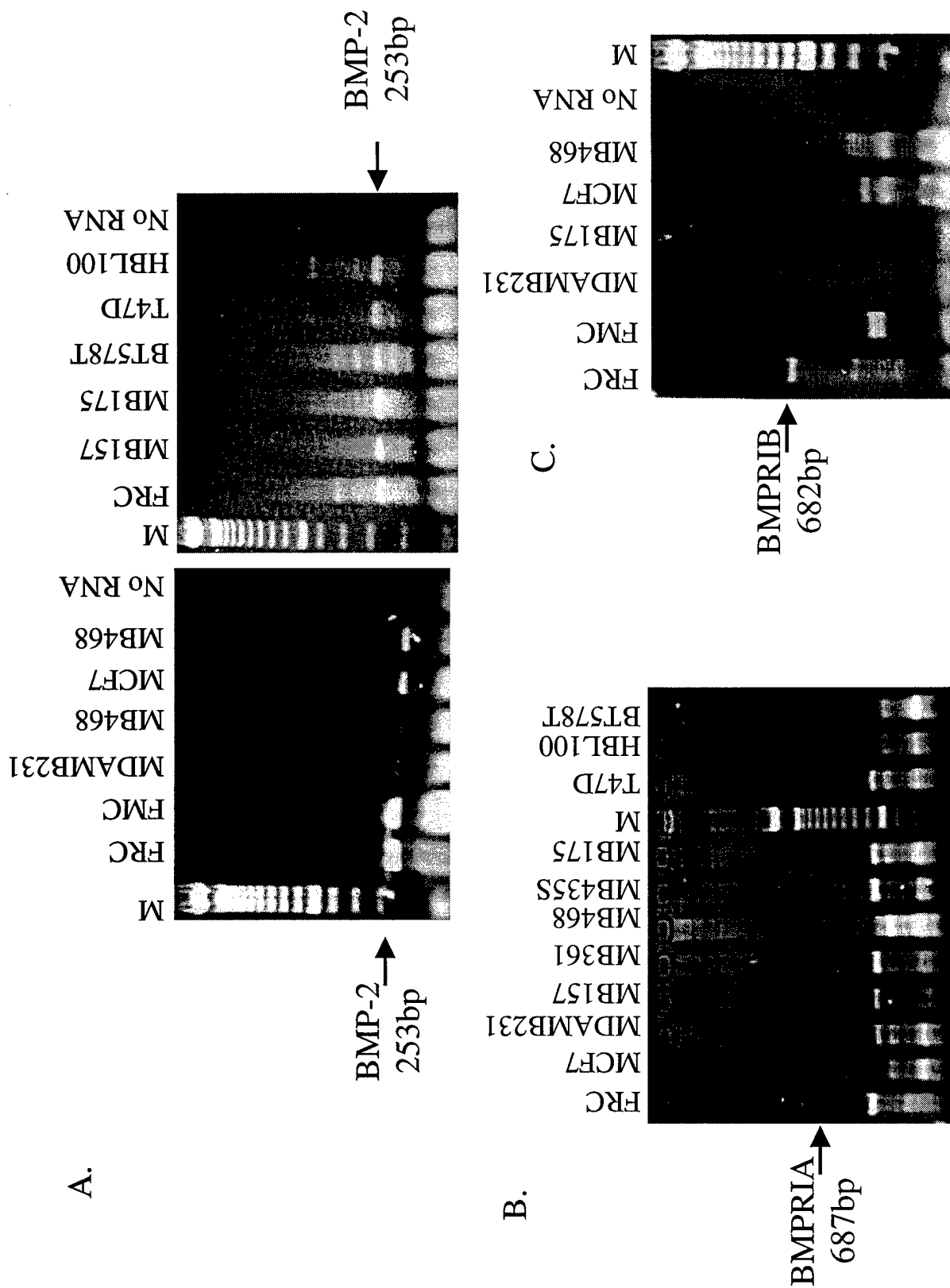
FIGURE 1. RTPCR detection of expression for BMP-2 (A), BMP receptor IA (B) and BMP receptor IB (C) mRNA in breast cancer cell lines. Total RNA was isolated from these cell lines and were subjected to reverse transcription followed by PCR reactions using primers to detect respective amplified DNA. The positions and sizes of the expected fragments are indicated in each figure.

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Towards this goal, attempts are now being made to standardize an assay model in animals for scoring the metastatic potential of a couple of these cell lines. I have not started collecting the tissue samples yet. This part is experiencing some delay due to the relocation of our Division of Oncology to Baylor college of Medicine at Houston. However I am in the process of communicating with them to get the tissue samples and their metastatic history from the tissue bank. Once that information is gathered we will test a few of those cell lines in our in vivo animal model, which will be standardized by then.

***Task 2.* To prove the clinical importance of BMP-2 expression and bone metastasis of breast cancer cells by genetically engineering the BMP-2 status of breast cancer cells and study the metastatic phenotype of the altered cells using an *in vivo* model of metastasis (months 6-24).**

- **Genetical alteration of the BMP-2 protein and receptor status (in cells chosen based on the results of task 1) by using stable transfection of corresponding cDNAs. These experiments will be initiated simultaneously with some of the experiments described in the Task 1. This period will be necessary to establish all the clonal cell lines and analyze their BMP protein and receptor expression profiles and finally to test the chemotactic properties of these cells . As the cell lines will be available, they will be tested for their metastatic potential by the in vivo assay in the animal model described above (months 6 - 24).**

In an attempt to express BMP-2 in breast cancer cell lines, in a regulatable manner, I have used a tetracyclin-inducible expression system as described by Gossen et al (Science, 268, 1766-1769, 1995). For this, I used breast cancer cell lines without estrogen receptor (ER), namely, MDA 435A. These cells were stably transfected with an expression plasmid for VP16 linked to a tetracyclin binding protein (pUHD 172-1). Stable clones were selected in 1mg/ml G418 and was tested for expression of rtTA by transient transfection with a reporter plasmid (pUHD 16-3) that consisted 7 tetracyclin operator sequences upstream of a luciferase gene. Treatment of cells with an analog of tetracyclin (doxacyclin) at 1μg/ml for 24hours showed inducible luciferase expression (2-10 fold) in several clones. The stable clones with the highest inducibility (MDA-435rtTA1) were then stably transfected with an expression plasmid containing BMP-2 cDNA under the control of a tet-inducible CMV promoter (pUHDBMP-2). After transfection, these cells were selected in 150 μg/ml hygromycin and analyzed for inducible BMP-2 expression by western blot analysis.

The results of western blot analysis showed expression of BMP-2 in all the clones of MDA-BMP-2 stable lines (Fig. 2). However we did not see much of an induction by doxacyclin treatment. Despite of this anomalous result, one of the MDA-BMP-2 clones (MDA-BMP-2-7) were analyzed for its growth property, in the presence and absence of doxacyclin, using direct cell counts (by MTT assay) and also by FACS (fluorescence activated cell sorting) analysis. As shown in Fig. 3, using MTT assay, the growth of MDA-BMP-2-7 cells were found to be significantly slower from day 5 of doxacyclin treatment. Using FACS analysis, which gives a measure of cell population undergoing cell division (i.e., cells entering the S phase of the cell cycle), we show that 48h doxacyclin treatment of MDA-BMP-2-7 cells reduced their S-phase entry by 3% compared to untreated control cells

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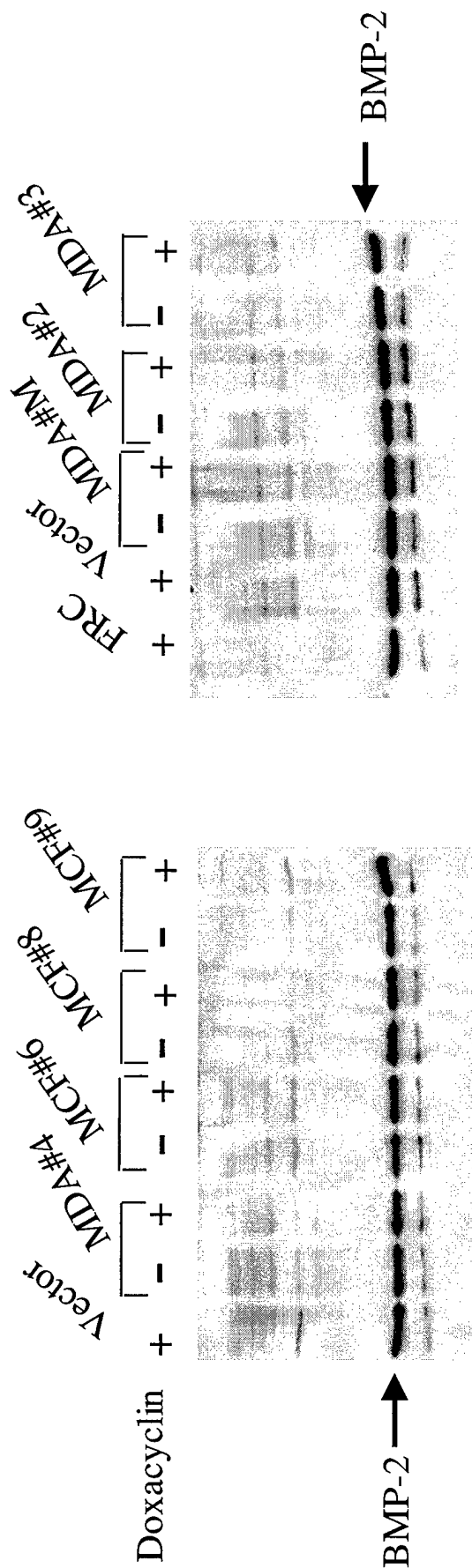
FIGURE 2. Analysis of MDAMB231 cells stably transfected with BMP-2 expression plasmid under tetracyclin regulatable promoter. Cell lysates were prepared in the after the cells were incubated with 1 μg/ml doxacyclin (+) or left untreated for 48h. The lysates were then analyzed for BMP-2 using anti BMP-2 antibody (Genetics Institute) in a Western analysis.
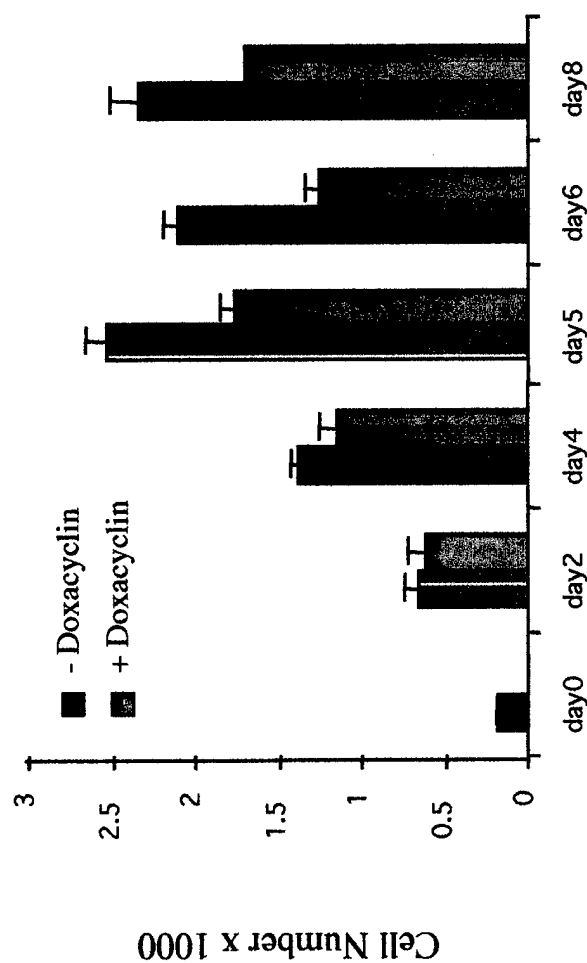
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FIGURE 3. MDA MB231 cells (clone 7) stably transfected with tetracyclin regulatable BMP-2 expression plasmid were analyzed for growth kinetics in the presence and absence of 1μg/ml doxacyclin(tetracyclin analog). Cell number was determined at days 0, 2, 4, 5, 6 and 8 using MTT method(see manuscript in the appendix).

(Table 1). Doxacyclin treatment also induced G1 arrest of these cells (Table 1). This suggests that doxacyclin treatment is inducing increased BMP-2 synthesis, which in turn is inhibiting growth of these cells. I am currently trying to make other stable cell lines by transfecting breast cancer cells with BMP receptors IA and IB expression plasmids. I am also in the process of generating similar cell lines using estrogen receptor positive MCF-7 as the parental cell line.

The methods used in the experiments for this section is described in details in the manuscript provided in the Appendix. This manuscript is accepted for publication in the Biochimica et Biophysica Acta (BBA) journal.

Table1. Cell cycle analysis of MDA MB 231 cell clone #7, trasfected stably with tetracyclin regulatable BMP-2 expression plasmid. The cells were treated with 1 μg/ml doxacyclin for 48h (+ Doxacyxclin) or were left untreated (- Doxacyclin) before fixing and labeling them with propidium iodide. The cells were then subjected to FACS analysis (described in details in the manuscript given in the Appendix). The percentage of the cells present in S or the G1 phases of the cell cycle were given in the following table.

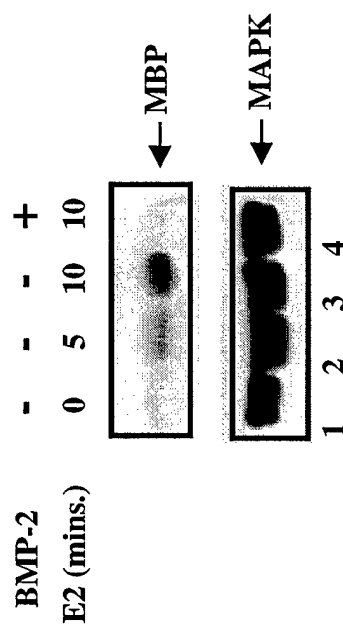|	- Doxacyclin	+ Doxacyclin
% of cells in S Phase	15.82	12.62
% of cells in G1 Phase	74.37	77.60

***Task 3*. To study differential effect of BMP-2 on ER-positive and ER-negative breast cancer cell growth and to investigate the underlying mechanism (months 6-18).**

- **Study effect of BMP-2 on ER-negative breast cancer cells *in vitro* by Flowcytometric analysis of cell cycle progression (months 6-8).**
Study the underlying mechanism of BMP-2 induced growth regulation of breast cancer cells. This part will include determination of the effect of BMP-2 on the MAPK pathway by enzymatic activity assay for MAPK, studying growth kinetics of breast cancer cells in the presence of MEK inhibitor and identification of other targets for BMP-2 action in these cells (months 9-24).

This part of the work was completed ahead of schedule. Please see attached manuscript in the Appendix, which gives the details of this part of the work and has been accepted for publication in BBA (MCF-7 cells). Here we show the mechanism by which BMP-2 inhibits estradiol-induced growth of MCF-7 cells. We found that BMP-2 increased p21 expression, inhibited cyclin dependent kinase (cdk) activities and finally inhibited phosphorylation of pRB. This resulted in hypophosphorylated pRb which was inhibitory to cell cycle progression and thus leads to decreased rate of cell proliferation. We also show that BMP-2 inhibits estradiol-induced MAPK activity in MCF-7 cells (Fig.4). Taken together, our data suggests that BMP-2 could be a very potent growth inhibitory agent as it can inhibit both nuclear and cytoplasmic events regulating cell proliferation.

A.

BMP-2   -   -   -   +

E2 (mins.)   0   5   10   10

← MBP

← MAPK

1   2   3   4

B.

Relative Luciferase Activity (Arbitrary Units)

300 — 250 — 200 — 150 — 100 — 50 —

Estradiol   -   +   -   +
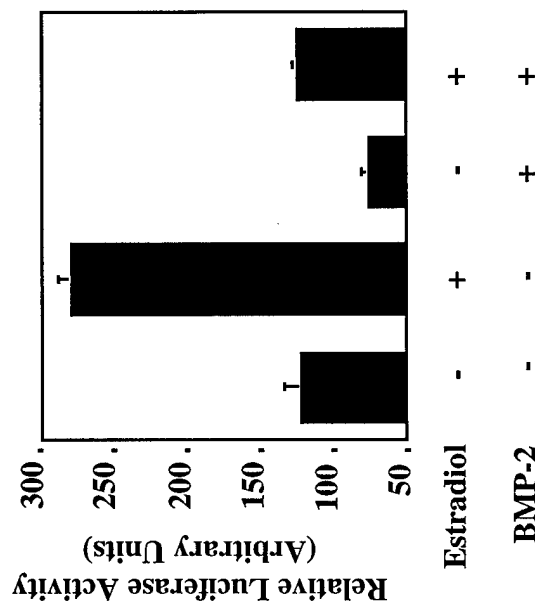
BMP-2   -   -   +   +

FIGURE 4. Inhibition of estradiol-induced mitogen activated protein kinase (MAPK) activity by BMP-2 in MCF-7 breast cancer cell line. MCF-7 cells treated with estradiol in the presence or absence of BMP-2. MCF-7 cells treated with estradiol in the presence or absence of BMP-2 were harvested. Cell lysates were immunoprecipitated with MAPK antibody and MAPK activity was measured in the immunecomplex beads using myelin basic protein (MBP) as a substrate. Panel A shows the autoradiogram of phosphorylated MBP (upper panel) and western analysis using MAPK antibody (lower panel), a measure of loading control. Panel B: Transactivating domain of MAPK-activated transcription factor Elk1 fused to yeast Gal4 DNA binding domain is cotransfected with Gal4 DNA binding elements linked to luciferase reporter gene, in MCF-7 cells. The luciferase activity was measured in cells treated with estradiol in the presence or absence of BMP-2. Estradiol activates MAPK, which in turn phosphorylates Elk. Elk1 activates expression of luciferase reporter gene in absence of BMP-2. Relative luciferase units were graphically shown in this panel. It shows that BMP-2 inhibits luciferase activity induced by estradiol treatment in these cells by inhibiting MAPK.

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Task 4. **To correlate the growth inhibitory effect of BMP-2 on ER-positive breast cancer cells *in vivo* (months 18-36).**

- **To study autocrine BMP-2 action (*in vitro*) : Analysis of effect of estradiol on growth kinetics of MCF-7 cells stably transfected with BMP-2 cDNA (months 18-24).**
- **To study autocrine BMP-2 action (*in vivo*) : Xenograft tumor formation assay of MCF-7 cells stably transfected with BMP-2 cDNA or vector alone (months 24-36).**

We have not started this part of the project yet. However, the standardization of the in vivo work that is currently going on in our laboratory will also be used for this part of the project.

Key Research Accomplishments:

- Identification of BMP-2 as a potential in vitro growth inhibitor for the breast cancer cells irrespective of their estrogen responsiveness.
- Understanding of the underlying mechanism of BMP-2-induced inhibition of breast cancer cell growth in vitro.

Reportable Outcome:

- **Abstract:** BMP-2 inhibits EGF-induced MDA-MB231 breast cancer cell growth via increased expression of p21 WAF1 cyclin kinase inhibitor (CKI) by posttranscriptional mechanism. N. Ghosh-Choudhury, S.L. Abboud and G. Ghosh-Choudhury. American Society of Bone and Mineral Research 21st Annual meeting, 1999. **(Poster Presentation)**
- **Abstract :** Mechanism for inhibitory role of BMP-2 on growth of estrogen-responsive and non-responsive human breast cancer cells. N. Ghosh-Choudhury, S.L. Abboud and G. Ghosh-Choudhury. International Conference Bone Morphogenetic Proteins 2000. **(Oral Presentation)**
- **Manuscript:** Bone morphogenetic Protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol treated MCF-7 human breast cancer cells. Nandini Ghosh-Choudhury, Goutam Ghosh-Choudhury, Anthony Celeste, Paramita M. Ghosh, Marissa Moyer, Sherry L. Abboud and Jeffrey Kreisberg. **Biochimica et Biopysica Acta, In Press.**

Conclusions:

We have started analyzing the role of a novel protein in the breast cancer cell growth mechanism. This protein, known as bone morphogenetic protein-2 (BMP-2) has been characterized before as a modulator of bone cell growth. We show that BMP-2 can significantly inhibit the growth of breast cancer cells *in vitro.* We have studied this growth inhibitory role of BMP-2 in estrogen-responsive and non-responsive breast cancer cells and

have found that it inhibits the growth of both the cell types with equal potency. In estradiol-responsive human breast cancer cells, BMP-2 can inhibit estradiol-induced growth of these cells. We have also identified the mechanism by which BMP-2 inhibits the growth of these cells (BBA, in press). Currently we are developing *in vivo* model system to test this *in vitro* phenomenon. If BMP-2 is found out to be as effective *in vivo*, then BMP-2 will prove to be a clinically important molecule for the breast cancer patients.

APPENDIX

Manuscript

Bone morphogenetic Protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol treated MCF-7 human breast cancer cells.

Nandini Ghosh-Choudhury, Goutam Ghosh-Choudhury, Anthony Celeste, Paramita M. Ghosh, Marissa Moyer, Sherry L. Abboud and Jeffrey Kreisberg.

Biochimica et Biopysica Acta, In Press.

Bone Morphogenetic Protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol treated MCF-7 human breast cancer cells

Nandini Ghosh-Choudhury[1*], Goutam Ghosh-Choudhury[2#*], Anthony Celeste[3], Paramita M. Ghosh[1], Marissa Moyer[1], Sherry L. Abboud[2#] and Jeffrey Kreisberg[1#]

Departments of [1]Pathology and [2]Medicine, The University of Texas Health Science Center at San Antonio; [#]GRECC, South Texas Veterans Health Care System, San Antonio, Texas; [3]Genetics Institute, Boston, MA.

Direct all correspondence to:
Nandini Ghosh-Choudhury
Department of Pathology
The University of Texas Health Science Center
7703 Floyd Curl Drive
San Antonio, Texas 78284-7750

Phone: 210-567-3108

FAX: 210-567-2303

e-mail: choudhury@uthscsa.edu

*These authors contributed equally to this work.

Running Title : BMP-2 inhibits breast cancer cell growth.

Key Words: BMP-2; pRb; p21, Breast cancer cells.

ABSTRACT

The biologic effects and mechanisms by which bone morphogenetic proteins (BMPs) function in breast cancer cells are not well defined. A member of this family of growth and differentiation factors, BMP-2, inhibited both basal and estradiol-induced growth of MCF-7 breast tumor cells in culture. Flow cytometric analysis showed that in the presence of BMP-2, 62% and 45% of estradiol-stimulated MCF-7 cells progressed to S-phase at 24 h and 48 h respectively. Estradiol mediates growth of human breast cancer cells by stimulating cyclins and cyclin-dependent kinases. BMP-2 significantly increased the level of the cyclin kinase inhibitor, p21, which in turn associated with and inactivated cyclin D1. BMP-2 inhibited estradiol-induced cyclin D1-associated kinase activity. Also estradiol-induced cyclin dependent kinase 2 (CDK-2) activity was inhibited by BMP-2. This inhibition of CDK activity resulted in hypophosphorylation of pRb thus keeping it in its active form. These data provide the first evidence by which BMP-2 inhibits estradiol-induced proliferation of human breast cancer cells.

1. INTRODUCTION

17-β-estradiol (estradiol) acts as a potent mitogen for breast epithelial cells and thus causes increased cell growth, both *in vivo* and *in vitro*. In estradiol responsive human breast cancer cells like MCF-7, the hormone responsiveness is mediated by estrogen receptors (ER) [1]. Activation of estrogen receptor stimulates cyclin-dependent kinases to induce proliferation of MCF-7 cells. On the other hand, increased expression of cyclin kinase inhibitor p21 blocks cyclin-dependent kinase activity necessary for pRb phosphorylation [2]. These results indicate that a concerted effect of different cell cycle proteins regulate cell cycle progression.

Bone morphogenetic proteins (BMPs), BMP 1-7, constitute a group of growth factors that are involved in ectopic bone formation [3]. They are produced as pro-mature forms, which are processed to active dimers of the mature region in a manner, similar to the transforming growth factor β (TGFβ) [4]. Due to this similarity, BMPs are categorized as members of the TGFβ super family. In addition to other functions during embryonic development and limb formation, BMPs regulate chondrogenesis and osteogenesis [5-7]. Though BMPs have been detected in osteosarcomas and soft tissue carcinomas, the role of BMPs in breast cancer is still unclear. Like TGFβ receptors, multiple BMP receptors have recently been identified. They form two closely related groups known as Type I and Type II receptors, which contain multiple members. Both the receptor types have serine/threonine kinase activity in their cytoplasmic domains [8,9]. Three downstream target molecules for BMP-2, have recently been identified. These targets are Smad 1, Smad 5 and Smad 8. BMP-2 stimulates association of Smad 1 with the BMP receptor followed by phosphorylation of the Smad 1 C-terminus by the type I receptor [10,11]. Receptor-phosphorylated Smad 1 undergoes heterodimerization with the tumor suppresser protein Smad 4. This heterodimer then translocates to the nucleus and participates in transcription of genes [10,12].

Recently Nakaoka et al demonstrated that BMP-2 inhibits smooth muscle cell proliferation [13]. BMP-2 blocks serum and androgen-induced growth of human prostate cancer cells in culture [14]. We have recently shown that BMP-2 at a moderate dose blocked PDGF and EGF-induced DNA synthesis in primary glomerular mesangial cells without any effect on matrix gene expression [15,16]. Also high dose of recombinant BMP-2 has recently been shown to inhibit soft agar growth of a variety of tumor samples including breast tumor [17]. However, the mechanism of BMP-2 mediated inhibition of tumor cell growth is not known. In this report, we demonstrate the inhibitory effect of BMP-2 on estradiol-induced MCF-7 human breast cancer cell proliferation in culture. BMP-2 increases the levels of cyclin kinase inhibitor p21 without any effect on estradiol-induced cyclin D1 expression. We also show that BMP-2 inhibits estradiol-induced cyclin D1-associated kinase and CDK2 activity with concomitant reduction of pRb phosphorylation. This is the first elucidation of the signaling mechanisms, involved in BMP-2-mediated inhibition of estradiol-induced breast cancer cell growth.

2. MATERIALS AND METHODS

Tissue culture materials were purchased from Gibco. Estradiol, PMSF, soybean inhibitor, leupeptin, myelin basic protein, propidium iodide and RNase A were obtained from Sigma. Histone H1 was purchased from Boehringer Mannheim. GST-pRB was obtained from Santa Cruz. Micro BCA reagent and ECL kit were purchased from Pierce. Protein A sepharose CL 4B was purchased from Pharmacia. All antibodies were obtained from Santa Cruz. Recombinant BMP-2 was obtained from Genetics Institute.

MCF-7 breast cancer cells were obtained from Dr. Robert Klebe (Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio) and were routinely maintained in DMEM-F12 medium containing 10% new born calf serum. These MCF-7 cells are highly responsive to estradiol and tamoxifene. For experiments, designed to test the mitogenic effect of estradiol, cells were grown in complete medium for 48 hours to reach subconfluency and then placed in phenol red-free and serum-free DMEM for 48 hours before addition of estradiol. Treatment with serum-free medium slows the growth of cells because they tend to arrest at G0/G1 phase. For cell cycle analysis near confluent cells were used for serum-deprivation to arrest in G0/G1 phase before addition of estradiol to release them.

2.1 *Flow cytometric analysis* : MCF-7 cells were trypsinized and washed with PBS. The cells were fixed in 70% ethanol for 30 minutes at -20^0C, centrifuged at 1500 x g for 4 minutes, washed with PBS containing 1% BSA and resuspended in 150 µl PBS. For nuclear staining with propidium iodide, the cells were treated with 50 µl of 1 mg/ml RNase A (SIGMA) followed by 100 µl of 100 µg/ml propidium iodide. The cells were incubated at 4^0C for 18 to 24 hours before they were analyzed by flow cytometry on FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA.). Cells were illuminated with 200 mW of light at 488 nm produced by an argon-ion laser and the fluorescence was read using a 630/22 nm band-pass filter. Data were analyzed for 20,000 viable cells as determined by forward and right angle light scatter and were stored as frequency histograms and subsequently analyzed by MODFIT software (Verity, Topsham, ME.).

2.2 *MTT assay for cell proliferation*: Proliferation of MCF-7 cells in response to estradiol was determined using the MTT assay as described elsewhere [18]. In brief, 50 µl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide) was added to the culture medium of growing cells (1 ml medium/well) and incubated for 4 hours at 37^0C in a humidified atmosphere with 5% CO_2. The medium was removed and 200 µl of DMSO was added to each well. The absorbance of the dissolved dye was measured at 540 nm.

2.3 *Immunoprecipitation and immunoblotting*: Immunoprecipitation was carried out according to methods described elsewhere, with minor modifications [16,19]. In brief, cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM β glycerophosphate, 50 mM NaCl, 0.1% Nonidet P-40, 50 µg/ml phenyl methyl sulphonyl fluoride, 10 µg/ml soybean trypsin inhibitor, 2 µg/ml leupeptin, 1µg/ml aprotinin) and cleared of cell debris by centrifugation at 4°C. Protein estimation was done in supernatant by a micro BCA assay. 200 µg protein was routinely precleared by incubating with 20 µl of swelled protein A-Sepharose beads for 1 h in the cold. The cleared supernatant was immunoprecipitated at 4°C for 18-24h using 1 µg of antibody followed by addition of 20 µl swelled protein A-Sepharose beads for 1 h. The protein A-Sepharose beads containing the antigen-antibody complex were then gently washed 3 times with immunoprecipitation buffer before eluting the bound proteins in the SDS polyacrylamide gel loading buffer.

Immunoblotting was performed essentially as previously described [20]. Briefly the cleared cell lysates or immunoprecipitates were separated in SDS-polyacrylamide gels (12% or 7.5%

depending on the protein sizes). The proteins were electrophoretically transferred onto Nytran membrane. Following the transfer, the proteins were incubated with blocking solution (50 mM Tris-HCl pH 7.4-150 mM NaCl-0.2% TWEEN 20 (TBST) containing 5% non fat dry milk) for 1 h at room temperature, followed by overnight incubation in primary antibody solution prepared in TBST containing 1% BSA. The membrane was subsequently washed 5 times in TBST for 5 minutes each, before the HRP conjugated secondary antibody was added in TBST for 1 h at room temperature. The membrane was finally washed in TBST, 5 times for 5 minutes each and the antigen-antibody complex was detected using an enhanced chemiluminescence kit (PIERCE) as per manufacturer's recommendations.

2.4 *Cyclin D1-associated kinase and CDK2 assay* : The assay was performed using the method of Gong et al [21]. Briefly, cleared cell lysate was immunoprecipitated using antibody against cyclin D1 or CDK2 as described above. The immunecomplex beads were resuspended in kinase buffer (20 mM Tris-HCl, pH 7.5 and 4 mM $MgCl_2$). To measure the cyclin D1-associated kinase activity, a fragment of pRb, that contains the *in vivo* phosphorylation sites, was used. For CDK2 activity, calf thymus histone H1 was used as a substrate. The reaction was carried out in the presence of 25 μM "cold" ATP and 10 μC$_i$ γ–^{32}P-ATP for 30 minutes at 37^0C. The phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Phosphorylation was quantitated using densitometric scan of the phosphorylated bands in autoradiogram.

3. RESULTS

Growth inhibition of MCF-7 cells by BMP-2 : Estradiol is a potent mitogen for ER positive human MCF-7 breast carcinoma cells [22]. To establish the optimal conditions to assess the effect of BMP-2 on MCF-7 cell proliferation, MCF-7 cells were treated with 1 nM 17β estradiol for 24 h and 48 h respectively and the cell number was counted. As expected, estradiol increased the cell number at each time point (Fig. 1A). To examine the effect of estradiol on cell cycle progression, MCF-7 cells were subjected to flow cytometry. At 24 h and 48 h, a significantly higher percentage of cells were in S-phase in the presence of estradiol, as compared to unstimulated control cells (Fig. 1B). At 48 h, 10% of cells entered into S-phase even in the absence of estradiol. This may be due to incomplete quiescence of MCF-7 breast tumor cells. An alternative possibility may be accumulation of mitogens in the culture medium during 48 h of incubation in the serum-deprived medium.

To determine the effect of BMP-2 on cell cycle progression of MCF-7 cells stimulated by estradiol, cells were incubated with estradiol for 24 h and 48 h, either in the presence or absence of BMP-2. BMP-2 inhibited estradiol-induced S-phase progression of these cells. Quantitation of these results show that only 62% and 45% of estradiol-treated MCF-7 cells entered S-phase at 24 h and 48 h respectively in the presence of BMP-2 (Fig. 1C, left and right panels). BMP-2 alone also inhibited S-phase entry of control cells, by 41% and 43% at 24 h and 48 h respectively. To determine if the effect of BMP-2 on estradiol-induced cell cycle progression correlated with cell growth, MTT assays were performed. Fig. 1D shows that BMP-2 significantly inhibited estradiol-stimulated as well as basal MCF-7 cell proliferation. A photomicrograph of MCF-7 cells in the absence and presence of BMP-2 is shown in Fig. 2. As evident treatment of these cells for 48 hours with BMP-2 does not have any toxic effect. Taken together, these results indicate that BMP-2 inhibits estradiol-induced cell growth by preventing the entry of MCF-7 cells into S-phase.

BMP-2 stimulates expression of cyclin kinase inhibitor, p21, in estradiol-treated MCF-7 cells: Progression of the cell cycle is regulated by a series of cyclin-dependent kinases (CDKs) [23]. These serine/threonine kinases are positively regulated by cyclins [24,25]. One of the G1 phase cyclins, cyclin D1, is overexpressed in more than 50% of human breast adenocarcinomas [26-28]. We studied the effect of estradiol on cyclin D1 expression. In accordance with the previous report [29], estradiol treatment of MCF-7 cells increased the level of cyclin D1 (Fig. 3A, compare lane 2 with lane 1). However, pretreatment of MCF-7 cells with BMP-2 had no significant effect on estradiol-induced expression of cyclin D1 (Fig.3A, compare lane 4 with lane 2). These data indicate that the effect of BMP-2 on estradiol-induced MCF-7 cell proliferation is not caused by the modulation of cyclin D1 levels during cell cycle progression.

CDK activity is also regulated by cyclin kinase inhibitors [25]. One such protein, p21, is a universal inhibitor of CDKs, that interacts with multiple cyclin-CDK complexes. It thereby inhibits their kinase activity, which drives the cells through the cell cycle [2,30,31]. To understand the mechanism of BMP-2 inhibition of cell cycle progression, we studied its effect on p21 expression. MCF-7 cells were incubated with BMP-2 and estradiol for 6 and 48 hours. At both time points, estradiol did not have any effect on p21 protein expression as determined by immunoblot analysis (Fig. 3B and 3C; compare lanes 2 with lanes 1 in both panels). In contrast, treatment of MCF-7 cells with BMP-2 alone significantly increased the level of p21 protein expression (Fig. 3B and 3C, compare lanes 3 with lanes 1). In the cells co-treated with BMP-2 and estradiol (lane 4), the level of p21 expression remained increased as compared to untreated and estradiol-treated cells. These data indicate that the inhibitory effect of BMP-2 on MCF-7 cell proliferation may partly be due to its effect on increased expression of p21.

BMP-2 inhibits cyclin D1-associated kinase activity via p21: For p21 to exert its inhibitory effect on cell cycle progression, it must associate with one of the cyclin-CDK complexes [24,25]. Since estradiol-induced increase in cyclin D1 levels resulted in cell cycle progression, while BMP-2-induced increase in p21 levels caused G1 arrest, we analyzed the association of p21 with cyclin

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D1 under similar conditions. Lysates of serum-deprived MCF-7 cells treated with estradiol in the presence and absence of BMP-2 were immunoprecipitated with the antibody to p21, followed by immunoblotting with a cyclin D1 antibody. As shown in Fig. 4A, estradiol alone has no effect on association of cyclin D1 with p21. In contrast, p21 was found to be associated with cyclin D1 in cells treated with BMP-2 alone or in combination with estradiol (Fig. 4A, lanes 3 and 4). These data indicate that BMP-2 treatment causes an increased association of p21 with cyclin D1 in MCF-7 cells which may result in the inhibition of cyclin D1-dependent kinase activity.

More recently a role for p21 has been described as the assembly factor for cyclin D and CDK4/6 [32,33]. Another role of p21 in cell cycle is its inhibitory effect on CDK activity. Increased expression of p21 has been shown to inhibit both Cyclin D1 and cyclin E associated kinases [2]. To test this, lysates of MCF-7 cells treated with BMP-2 and estradiol were immunoprecipitated with cyclin D1 antibody. The immunoprecipitates were assayed for D1-associated kinase activity using pRb as *in vitro* substrate. As shown in Fig. 4B, BMP-2 inhibited estradiol-induced cyclin D1-asssociated kinase activity (compare lane 2 with lane 1). These data indicate that inhibitory effect of BMP-2 may involve reduced pRb phosphorylation by cyclin D1-associated kinase (see below).

BMP-2 inhibits estradiol induced CDK-2 kinase activity and pRb phosphorylation: In the late G1 phase of cell cycle progression, E-type cyclin regulates CDK activity which is necessary for cells to enter into and proceed through the S-phase (34). p21 has been shown to regulate cyclin E via physical association. Since p21 level was increased by BMP-2 (Fig. 3B,C), we tested if recombinant BMP-2 regulates p21 association with cyclin E. Cyclin E immunoprecipitates from lysates of estradiol or BMP-2 plus estradiol-treated MCF-7 cells were immunoblotted with p21 antibody. The results show that BMP-2 stimulated increased association of p21 with cyclin E in the presence and absence of estradiol as compared to estradiol alone (Fig. 5A, compare lanes 3 and 4 with lane 2). During late G1 and S-phase cyclin E regulates CDK2 activity. To understand the mechanism of regulation of CDK2 in MCF-7 breast cancer cells, we analyzed the kinase activity associated with CDK2 in cells treated with estradiol in the presence or absence of BMP-2. Cell lysates were immunoprecipitated with a CDK2 antibody. The immunebeads were then used in an *in vitro* immunecomplex kinase assay with histone H1 as substrate in the presence of γ^{32}P-ATP. The data showed increased phosphorylation of histone H1 by CDK2 in cells treated with estradiol (Fig. 5B, compare lane 2 with 1). Estradiol-induced CDK2 activity was significantly inhibited by BMP-2 (Fig. 5B compare lane 4 with lane 2). Quantitation of histone H1 phosphorylation showed eleven fold increase in CDK2 activity in the presence of estradiol (Fig. 5C), and BMP-2 inhibited 70% of estradiol-induced CDK2 activity (Fig. 5C). BMP-2 also partially inhibited the basal activity of CDK2 in MCF-7 cells. These data indicate that BMP-2-mediated inhibition of estradiol-induced MCF-7 proliferation may in part be due to its inhibitory effect on CDK2 activity.

One of the targets of CDKs during cell cycle progression is pRb [24,35]. Hypophosphorylated pRb is active and inhibits cell cycle progression. Proliferative signals integrate into the nucleus to induce CDK-dependent phosphorylation of pRb rendering pRb inactive and resulting in DNA synthesis [35]. We showed in Fig. 4B that in *in vitro* kinase assay, pRb phosphorylation is reduced by BMP-2. To study the effect of BMP-2 on estradiol-induced pRb phosphorylation in MCF-7 cells, we analyzed lysates of MCF-7 cells by phosphorylation-dependent mobility shift assay. The degree of pRb phosphorylation is determined by its electrophoretic mobility, with hyperphosphorylated pRb forms migrating slower than the hypophosphorylated form in SDS gel. Lysates from MCF-7 cells, treated with estradiol or BMP-2 alone or with estradiol in the presence of BMP-2, were immunoblotted with an anti-pRb antibody. As shown in Fig 6, estradiol caused hyperphosphorylation of pRb as indicated by the slower migration of this protein (lane 1, indicated by filled circle). Treatment of MCF-7 cells with BMP-2 alone resulted in the partial phosphorylation of pRb (lane 2, indicated by open circle). In contrast, BMP-2 significantly inhibited hyperphosphorylation of pRb induced by estradiol and only hypophosphorylated pRb was the predominant form detected (Fig. 6, lane 3, indicated by filled

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triangle). These data indicate that the observed growth inhibitory effect of BMP-2 in estradiol-induced MCF-7 breast cancer cell proliferation could be caused by decreased CDK-dependent pRb phosphorylation.

4. DISCUSSION

Our study demonstrates an inhibitory effect of BMP-2 on estradiol-induced MCF-7 breast cancer cell proliferation. BMP-2 stimulates increased expression of p21 cyclin kinase inhibitor. Consistent with this idea is our observation showing inhibition of estradiol-induced cyclin D1-associated kinase and CDK2 activity in response to BMP-2. Finally, we provide the first evidence that BMP-2 maintains the pRb tumor suppressor protein in a partially phosphorylated form.

Binding of estrogen to its receptor regulates a cohort of responsive genes that appears to regulate cell cycle progression. CDK4 and CDK6 form complexes with D-type cyclins during mid and late phases of G1, while CDK2 binds to cyclin E and D during late G1 [36-38]. One link between proliferative signals and cell cycle progression is provided by the induction of the secondary response genes, such as cyclin D1, following mitogenic stimulation [36]. In breast cancer, chromosome 11q13, which contains the cyclin D1 gene, has been shown to be amplified preferentially in estrogen receptor positive tumors [39,40]. It has also been suggested that overexpression of cyclin D1 in MCF-7 cells causes them to proliferate in growth factor-deprived condition [41]. In simvastatin or lovastatin-arrested MCF-7 human breast cancer cells, estrogen stimulates cell cycle entry by increasing cyclin D1 expression [42]. This effect of estrogen was due to transcriptional activation of the cyclin D1 gene by an estrogen-regulated response region present between the -944 bp of upstream sequences and the transcription start site of the cyclin D1 gene [42]. Cyclins, in association with CDKs and cyclin kinase inhibitors, control cell cycle progression through different phases of transitions and checkpoints. One of the cyclin kinase inhibitors, p21, has been shown to stimulate withdrawal from the cell cycle coupled to terminal differentiation [43]. Immunohistochemical analysis of breast carcinomas has shown that increased expression of p21 was associated with relapse-free survival [44]. p21 inhibits all the CDKs associated with cyclins A, D1 and E that are required for G1/S progression [31,45]. In addition to CDK inhibition, and thereby blocking cells from entering into S-phase, p21 inhibits the DNA replication directly by binding to PCNA [46]. In the present study, we show that estradiol-induced S-phase entry of MCF-7 breast carcinoma cells is inhibited by the growth and differentiation factor BMP-2 (Fig. 1). Furthermore our results demonstrate that BMP-2 causes increase in the levels of p21 protein as early as 6 hours which sustains till 48 hours (Fig. 3B,3C). These data indicate that our observation of BMP-2-induced reduction in S-phase entry (Fig. 1C) and reduced proliferation (Fig. 1D) may be due to the increased expression of p21 protein (Fig. 3B,3C). One of the mechanisms by which p21 blocks cells from entering into S-phase is via interaction with cyclin D1 during G1 phase of the cell cycle, subsequently resulting in inhibition of CDK4 activity [2]. In the present study, we demonstrate association of p21 with cyclin D1 in the presence of BMP-2 (Fig. 4A). This may be the cause of reduced cyclin D1-associated kinase activity (Fig. 4B).

In addition to activation of cyclin D1/CDK4 during G1 phase, activation of cyclin E/CDK2 in late G1 is required for cells to progress through the cell cycle [38,47]. p21 inhibits both cyclin D1/CDK4 activity and cyclin E/CDK2 activity [2,30,48]. Treatment of MCF-7 breast cancer cells with estradiol stimulates cyclin D1-associated kinase (Fig. 4B) and CDK2 activity (Fig. 5B) which confirms the previous finding [47]. Pretreatment of cells with BMP-2, however, significantly blocked the estrogen-induced increase in both these kinase activities (Fig. 4B, and Fig. 5B). Furthermore, BMP-2 increased the association of p21 with cyclin E (Fig. 5A). Our data for the first time demonstrate that BMP-2 targets the cell cycle machinery at the level of cyclin dependent kinases. Thus one of the mechanisms by which BMP-2 inhibits MCF-7 cell proliferation is by inhibiting cyclin dependent kinases that are known to be activated in mid to late G1 and S phases of cell cycle.

One of the targets of G1 CDKs is the tumor suppresser protein pRb [35]. This notion is established from various *in vitro* and *in vivo* studies. Cyclin D1/CDK4 complex can phosphorylate pRb *in vitro* [49]. The physiologic regulators that intercept CDK4/6 activity also block pRb

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phosphorylation. Similarly, overexpression of cyclin E in human osteosarcoma cells increases pRb phosphorylation [50]. pRb is also hyperphosphorylated in various breast cancer cells and tissues by cyclin E/CDK2 activity [51]. In tamoxifen-arrested MCF-7 cells, estradiol stimulates cyclin E/CDK2-dependent pRb phosphorylation [47]. We have also shown that treatment of serum-deprived MCF-7 cells with estradiol increased the level of hyperphosphorylated inactive pRb and that presence of BMP-2 during estradiol treatment caused reduction in the degree of pRb phosphorylation (Fig. 6). These observations describe one of the first mechanisms by which BMP-2 may inhibit MCF-7 breast cancer cell growth in culture.

In summary, we have demonstrated that BMP-2 inhibits estradiol-induced proliferation of human breast cancer cells. This effect of BMP-2 appears to be mediated by inhibition of positive cell cycle regulatory proteins. Hyperproliferation of estrogen-responsive breast cancer cells is one of the major causes of tumor formation in early stages of breast cancer. Agents such as BMP-2 that inhibit estradiol-induced breast cancer cell proliferation may prove to be important therapeutic tools once their mechanisms of action are more thoroughly characterized.

ACKNOWLEDGMENT

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LEGENDS TO THE FIGURES

Figure 1. *Effect of estradiol and BMP-2 on MCF-7 cell proliferation.* (A) Confluent layers of MCF-7 cells in 35 mm tissue culture dishes were serum starved for 48 hours and incubated with 1 nM estradiol for 24 h and 48 hours. Cells in each dish were counted. Increase in cell number was 1.6 and 1.8 fold respectively at 24 and 48 hours. (B) *Flow-cytometric analysis of estradiol-induced MCF-7 cell proliferation.* Serum-deprived MCF-7 cells were grown in the presence or absence of 1 nM estradiol for 24 h and 48 hrs. Cells were then trypsinized and analyzed by flow cytometry as described in the Methods. The percentage of cells in S-phase was plotted. The percentage of cells in S-phase was increased by 6.5 and 3.7 fold at 24 and 48 hours respectively. Means of triplicate determinations are shown in A and B. (C) *Quantitation of flow cytometric analysis of cells treated with BMP-2 and estradiol.* MCF-7 cells were treated with 100 ng/ml BMP-2 and 1nM estradiol for 24h (left panel) and 48h (right panel) before subjecting them to Flow-cytometric analysis. The percentage of cells in S-phase was plotted for each condition. (D) *Effect of BMP-2 on estradiol-induced MCF-7 cell proliferation.* 48h serum-deprived MCF-7 cells were treated with 100 ng/ml BMP-2 and 1 nM estradiol. MTT assay was performed as an index of cell proliferation as described in Methods. Results are means ±SE of three independent experiments. *$P < 0.05$ vs. untreated cells. ++$P < 0.05$ vs. untreated control. @$P < 0.05$ vs. estradiol-treated cells.

Figure 2. Photomicrograph of MCF-7 cells in the presence and absence of BMP-2. Serum-deprived MCF-7 cells were incubated with BMP-2 for 48 hours before taking the photograph. The phase contrast photomicrograph is shown.

Figure 3. (A) *Effect of BMP-2 on estradiol-induced expression of cyclin D1 and p21[1].* MCF-7 cells serum-deprived for48 hrs, were incubated with 1 nM estradiol in the presence and absence of 100 ng/ml BMP-2 for subsequent 48 hrs. Cleared cell lysates were analyzed by immunoblotting with cyclin D1. (B and C) Effect of BMP-2 on p21 expression. Serum-deprived MCF-7 cells were incubated with estradiol in the presence and absence of 100 ng/ml BMP-2 for 6 hours (panel B) and 48 hours (panel C) respectively. The lysates were immunoblotted with p21 antibody and the signal was developed by ECL. Lower panels show immunoblotting of same lysates with anti-actin antibody to demonstrate equal loading of proteins in each lane.

Figure 4. (A) *Effect of BMP-2 on association of p21 with cyclin D1.* Cleared cell lysates from MCF-7 cells, treated as described in Fig. 3B and C, were immunoprecipitated (I.P.) with either anti-p21 or control IgG. The immunoprecipitated proteins were eluted from the immunebeads, separated on a 12% SDS-polyacrylamide gel and were immunoblotted (I.B.) with anti-cyclin D1 antibody. Lanes 1 through 4 represent p21 immunoprecipitates. Lane 5 shows IgG immunoprecipitates. (B) Effect of BMP-2 on estradiol-induced cyclin D1-associated kinase activity. Serum-deprived MCF-7 cells were incubated with estradiol in the presence of BMP-2. The lysates were immunoprecipitated with cyclin D1 antibody. The immunoprecipitates were assayed for kinase activity in the presence of γ^{32}P-ATP using pRb as substrate. The labeled protein was separated by SDS gel electrophoresis and visualized by autoradiography. The lower panel shows immunoblot of same samples with actin antibody.

Figure 5. (A) *Effect of BMP-2 on association of p21 with cyclin E.* Cleared cell lysates from MCF-7 cells, treated as described in Fig 4, were immunoprecipitated (I.P.) with anti-cyclinE. The immunoprecipitated proteins were eluted from the immunebeads, separated on a 15% SDS-polyacrylamide gel and were immunoblotted (I.B.) with anti-p21 antibody.

Effect of BMP-2 on estradiol-induced CDK2 activity. (B) The cleared cell lysates from MCF-7 cells treated as described in Fig. 4, were immunoprecipitated with an anti-CDK2 antibody. The washed immunebeads were used in an *in vitro* immunecomplex kinase assay with histone H1 as

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substrate in the presence of γ^{32}P-ATP. The labeled proteins were separated by SDS gel electrophoresis and visualized by autoradiography. The lower panel shows immunoblotting of the same lysates with anti-actin antibody. (C) *Quantitation of histone H1 phosphorylation.* The radioactivity incorporated into histone H1 in panel A was measured by densitometric scan as described in the Method and plotted as histogram.

Figure 6. *Effect of BMP-2 on estradiol-induced pRb phosphorylation.* The cleared cell lysates of MCF-7 cells, treated with estradiol in the presence and absence of BMP-2 were immunoblotted with an anti-pRb antibody. The migration of molecular weight markers (in kDs) is shown in left margin. The filled circle shows hyperphosphorylated pRb in highest phosphorylated form. The open circle shows pRb in intermediate phosphorylated form and the filled triangle shows pRb in hypophosphorylated form.
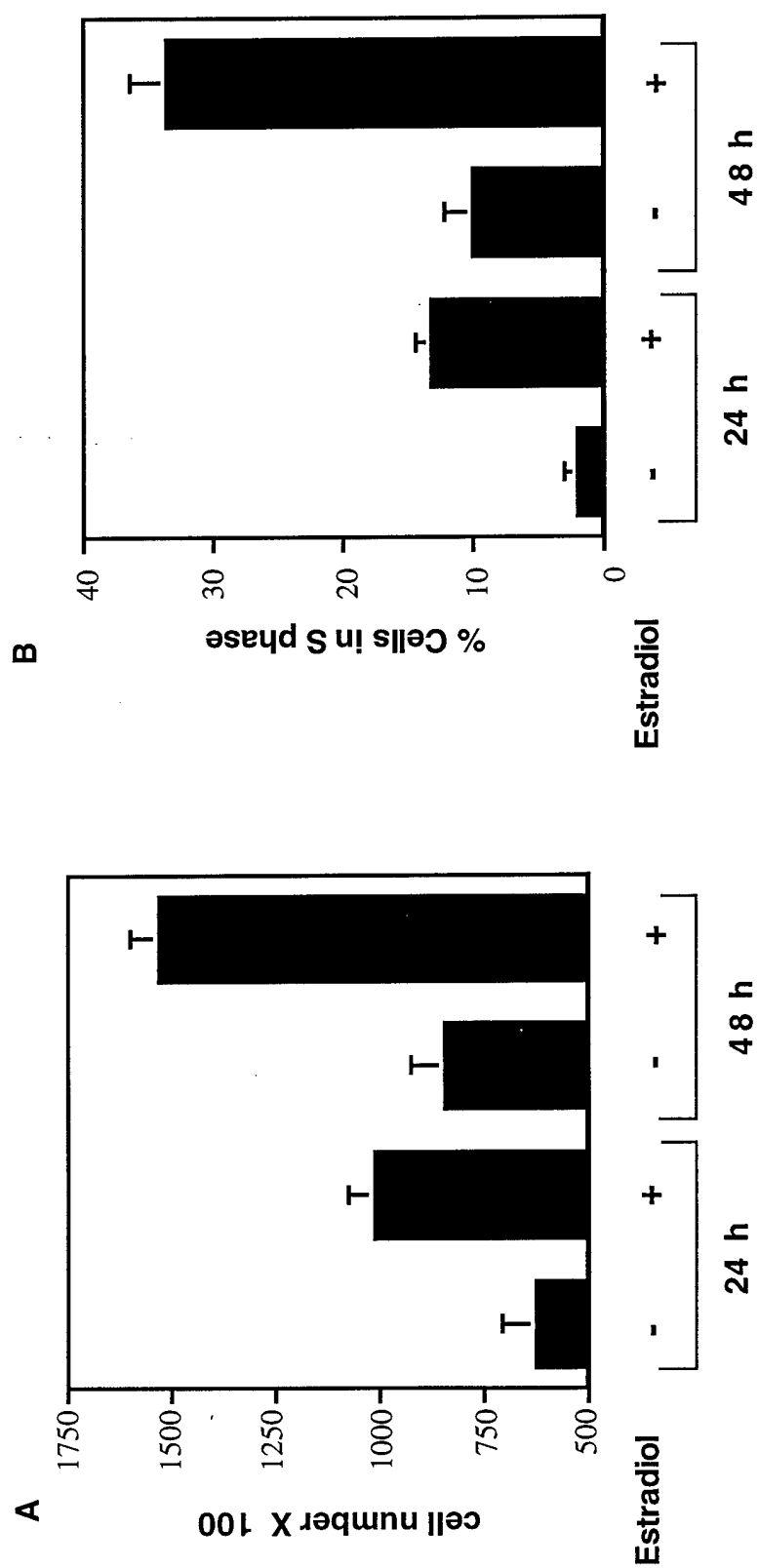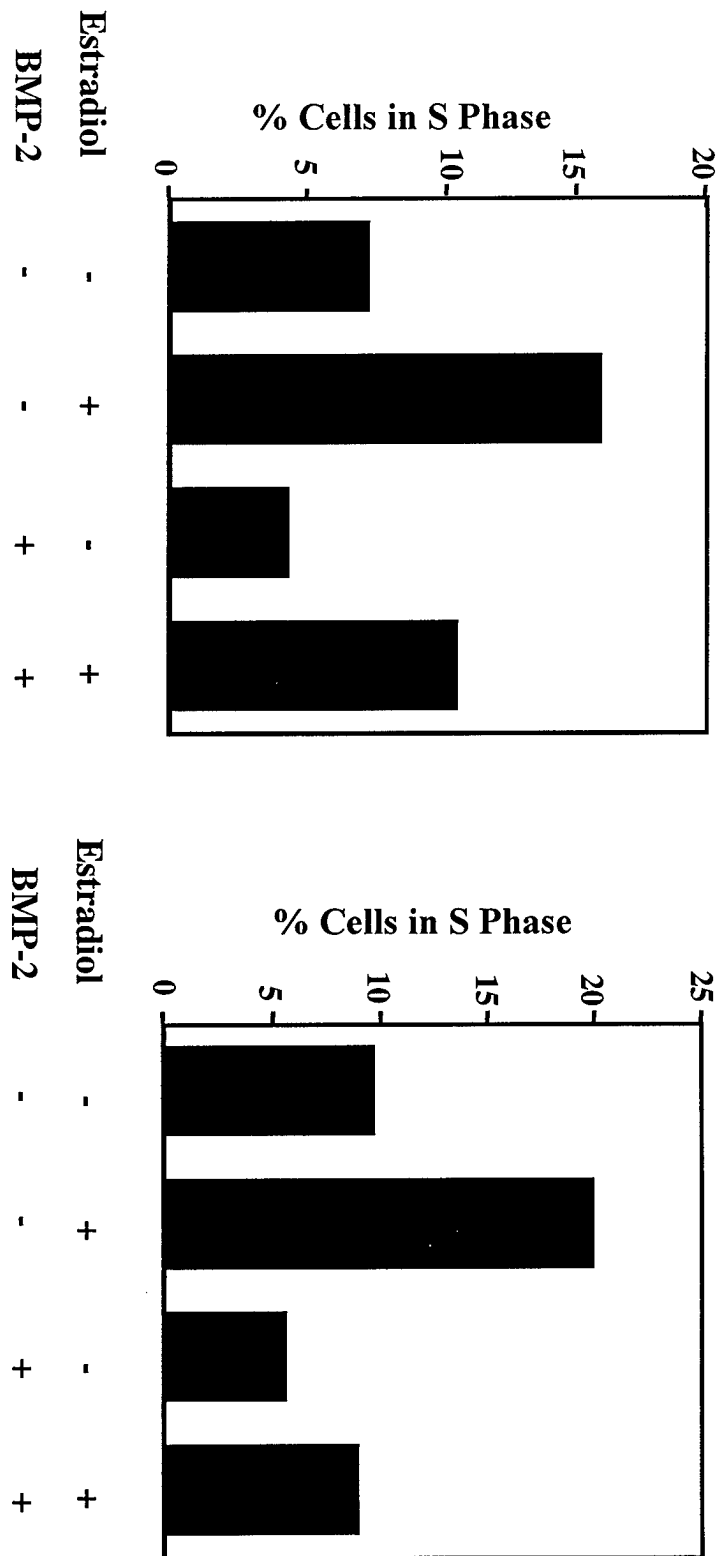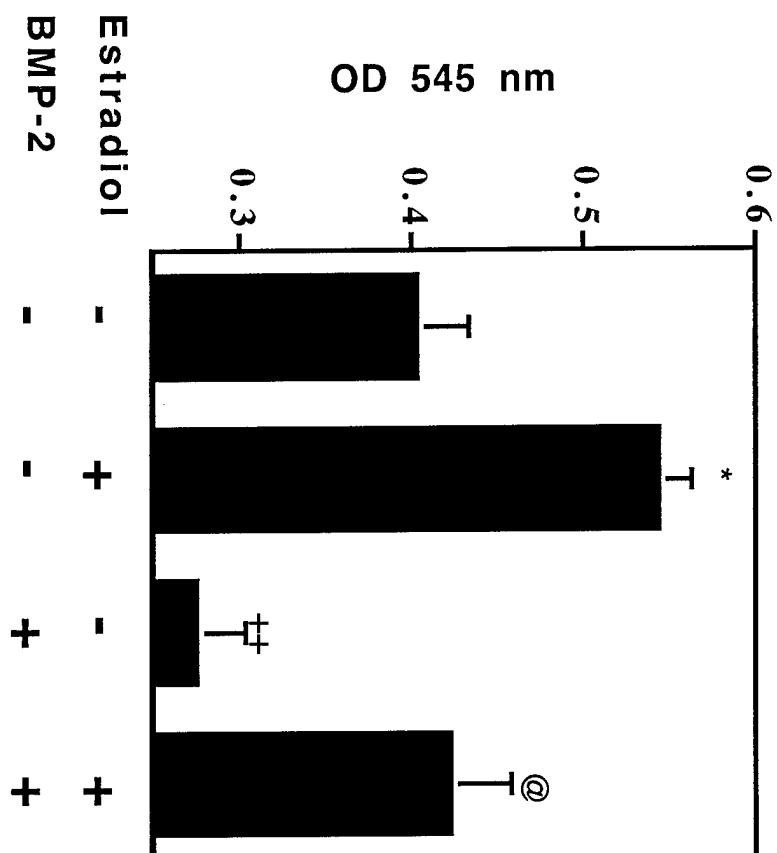
FIGURE 1

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C

% Cells in S Phase

Estradiol
BMP-2

% Cells in S Phase

Estradiol
BMP-2

FIGURE 1

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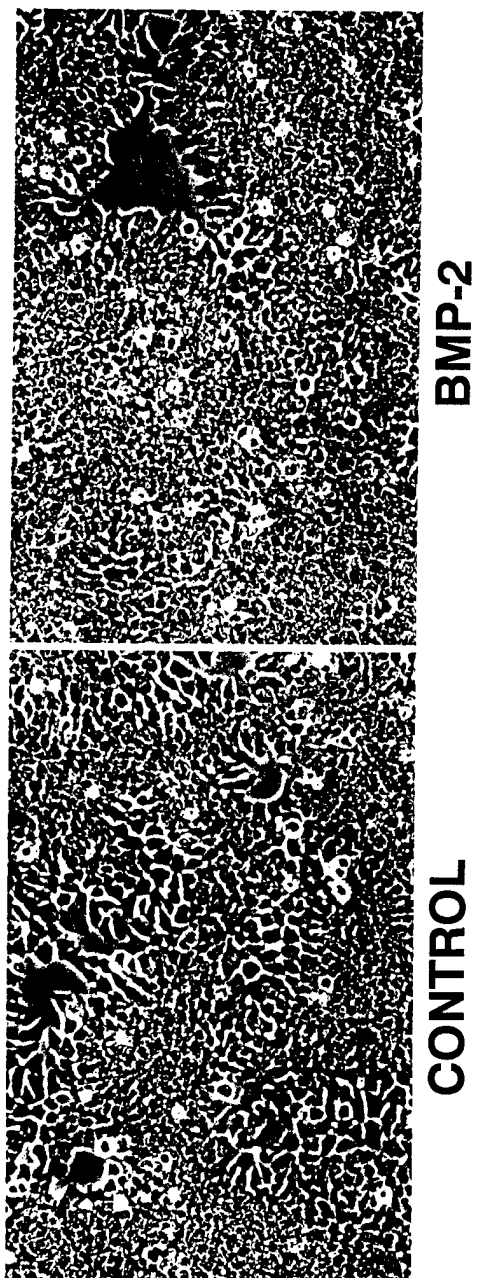
FIGURE 2

Ghosh-Choudhury et al

BMP-2
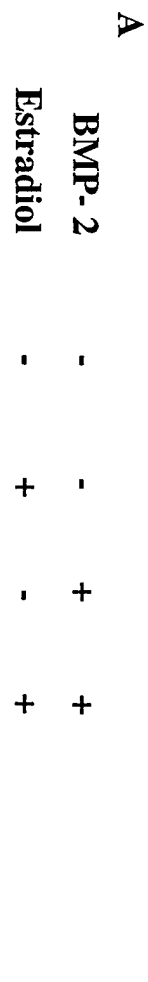
CONTROL

A

	BMP-2	Estradiol
	-	-
	+	-
	-	+
	+	+

← Cyclin D1

	1	2	3	4

← Actin

B

	1	2	3	4
BMP-2	-	+	-	+
Estradiol	-	-	+	+

← p21

← Actin

C

	1	2	3	4
BMP-2	-	+	-	+
Estradiol	-	-	+	+

43—
28—

← p 21

← Actin
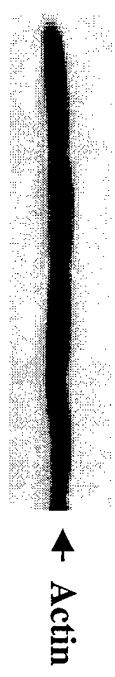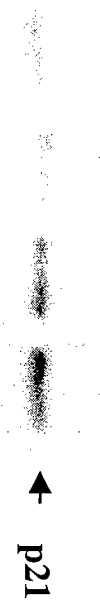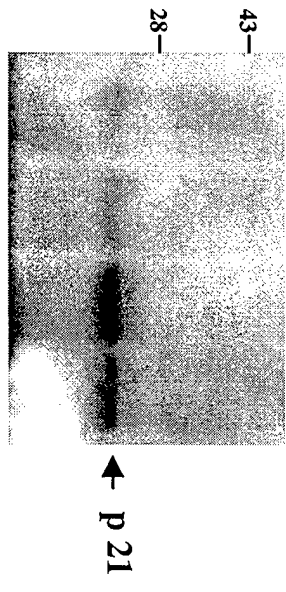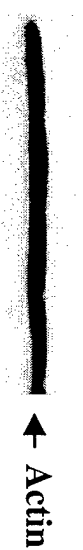
FIGURE 3

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FIGURE 4

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A

Antibody I. B.
Antibody I. P.

BMP-2
Estradiol

Cyclin D1

	p 21			IgG
-	-	+	+	+
-	+	-	+	+
1	2	3	4	5

← Cyclin D1

B

BMP-2
Estradiol

-	-	+	+	+
-	+	+	+	-

← pRb

◄ Actin

1	2	3

FIGURE 5

A.

I B Antibody

I P Antibody

BMP- 2

Estradiol

p21

	1	2	3	4
BMP- 2	-	-	+	+
Estradiol	-	+	+	-

cyclinE

◄ p21

B.

	1	2	3	4
BMP- 2	-	-	+	+
Estradiol	-	+	-	+

28 ─

19 ─

◄ Histone H1

1 2 3 4

◄ Actin

C.

CDK 2 Activity
(Arbitrary units)

Estradiol

BMP-2

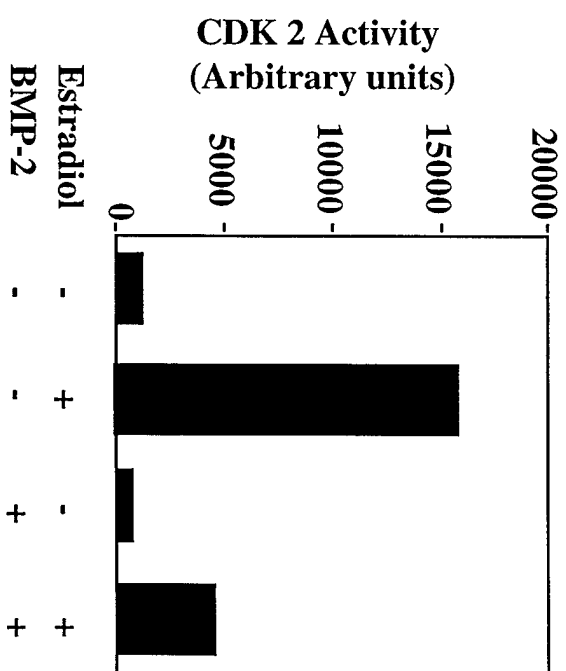|			
Estradiol	-	+	-	+
BMP-2	-	-	+	+

0 — 5000 — 10000 — 15000 — 20000 —
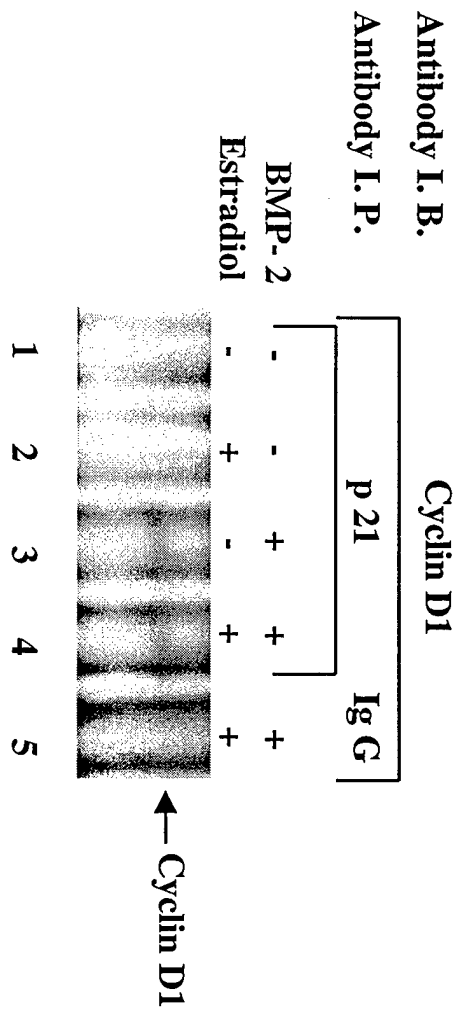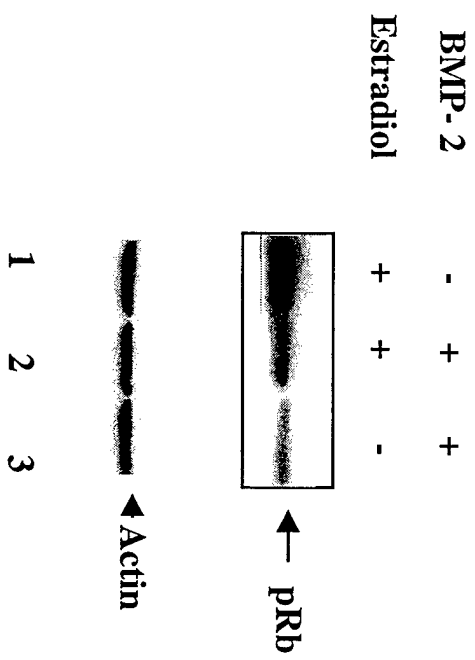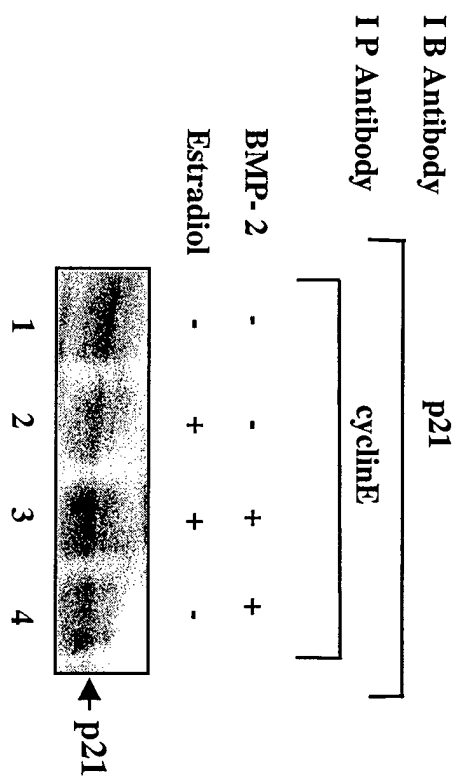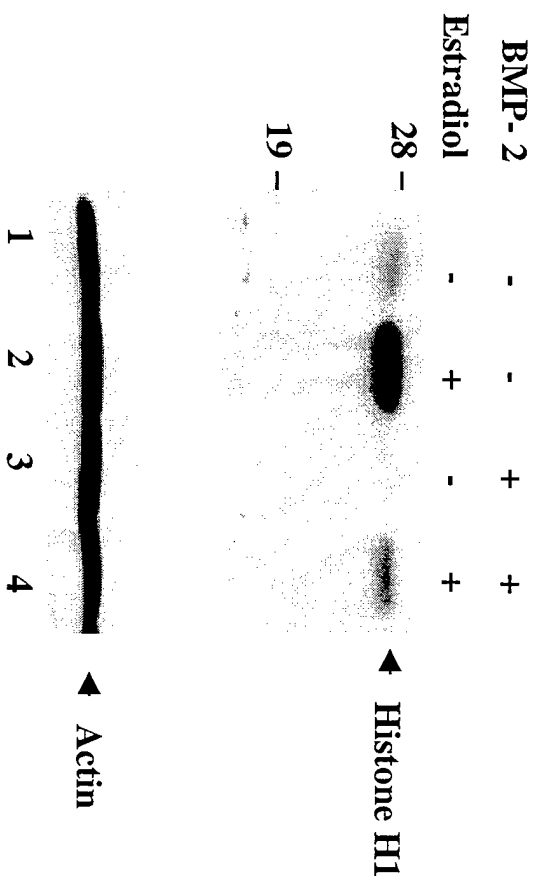
FIGURE 6

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BMP- 2

Estradiol

200 —

107 —

1 2 3

} ← p Rb

← Actin